ORIGINAL ARTICLE INFECTIOUS DISEASES

Streptococcus pneumoniae exposure is associated with human metapneumovirus seroconversion and increased susceptibility to *in vitro* HMPV infection

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Abstract

It remains largely unknown which factors determine the clinical outcome of human metapneumovirus (HMPV) infections. The aim of the present study was to analyse whether exposure to bacterial pathogens can influence HMPV infections. From 57 children, serum samples and colonization data for *Haemophilus influenzae*, *Moraxella catarrhalis*, *Staphylococcus aureus* and *Streptococcus pneumoniae* were collected at 1.5, 6, 14 and 24 months of age. Seroconversion rates to HMPV were determined and related to bacterial carriage. Frequent nasopharyngeal carriage (≥2 times in the first 2 years of life) of *S. pneumoniae*, but not of the other three pathogens, was associated with increased seroconversion rates of infants to HMPV at the age of 2 years (frequently vs. less exposed, 93% vs. 59%; $p < 0.05$). Subsequently, the susceptibility of well-differentiated normal human bronchial epithelial cells (wd-NHBE) pre-incubated with bacterial pathogens to *in vitro* HMPV infection was evaluated. Pre-incubation of wd-NHBE with *S. pneumoniae* resulted in increased susceptibility to infection with HMPV-enhanced green fluorescent protein (EGFP), as determined by enumeration of EGFP-positive cells. This was not the case for cells pre-incubated with *H. influenzae*, *M. catarrhalis* on *S. aureus*. We conclude that exposure to *S. pneumoniae* can modulate HMPV infection.

Keywords: Bacterial and viral interaction, human metapneumovirus (HMPV), pathogenesis, respiratory infection, *Streptococcus pneumoniae*
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Introduction

Acute respiratory tract infections (RTI) caused by bacterial or viral infections are responsible for considerable morbidity. Human metapneumovirus (HMPV), a member of the family *Paramyxoviridae*, is an important viral cause of RTI [1]. HMPV causes worldwide seasonal outbreaks of respiratory tract infections, in moderate climate zones predominantly during the winter season. The main risk groups for development of severe disease after HMPV infection are young infants, individuals with underlying disease and the elderly [2]. In patients with severe disease the main clinical diagnoses are bronchiolitis and pneumonia, occasionally leading to death [3].

In young infants HMPV infection appears to be ubiquitous, as virtually all children are seropositive by the age of 5 years. Estimates of the percentage of acute paediatric lower RTI associated with HMPV range from 5% to 25% [2]. However, it remains largely unknown which factors determine the clinical outcome of HMPV infections. It has been speculated that disease severity could be enhanced by bacterial superinfections. Indeed, bacterial pathogens such as *Streptococcus pneumoniae* can often be detected in patients with

HMPV-associated severe disease [4]. In addition, the incidence of invasive pneumococcal disease in children is associated with the seasonality of respiratory virus infections [5]. Furthermore, introduction of a multivalent pneumococcal conjugate vaccine resulted in reduction of pneumonias associated with respiratory virus infections [6]. In mice, HMPV infection was shown to influence subsequent superinfection with *S. pneumoniae* [7]. The aim of the present study was to analyse whether exposure to respiratory bacteria can influence HMPV infections.

Methods

Patients and samples

The study population consisted of 57 healthy children of the Generation R Focus Study [8]. Enrolled mothers were residents in the study area at their delivery date, which had to be between April 2002 and January 2006. Selection of eligible children for the present study was performed on the basis of availability of adequate quantities of both nasal swab samples for bacterial culture and serial serum samples for assessment of HMPV seroconversion. Samples were collected after written informed consent from parents or guardians. The study was carried out in accordance with human experimentation guidelines in the Netherlands, and approved by the medical ethical committee of the Erasmus MC.

For all 57 children, three or four serial serum samples had been collected over a period of 2 years: 54 (95%) cord blood serum samples and 32 (56%), 46 (81%) and 45 (79%) serum samples collected at the ages of 6, 14 and 24 months of age, respectively. A nasopharyngeal swab was obtained for cultivation of *Haemophilus influenzae*, *Moraxella catarrhalis* and *S. pneumoniae* and a nasal swab was obtained for cultivation of *Staphylococcus aureus*. Bacterial colonization data were collected at 1.5, 6, 14 and 24 months of age for 40 (70%), 49 (86%), 50 (88%) and 48 (84%) children, respectively. Children were classified as 'frequently exposed' if two or more swab cultures were positive for one of the bacterial species in the first 2 years of life. When a culture time-point was missing, this was considered negative. None of the patients had used antibiotics in the 48 h before swab collection. Microbiological analyses were performed as described previously [9]. All children were fully vaccinated according to the Dutch National Immunization Programme, including a vaccination with measles-mumps-rubella (MMR) around the age of 14 months. At the time this study was performed, vaccination for *S. pneumoniae* was not part of the National Immunization Programme.

Serology

Serum IgG levels specific for HMPV and measles virus (MV) were quantified using ELISA as described previously [10]. Briefly, 96-well plates were coated with inactivated MV or the recombinant fusion protein of HMPV. Serum samples were tested in duplicate in a 1 : 300 dilution; results are shown as mean OD-values after subtraction of background values. A greater than two-fold increase in OD450 resulting in a value above 0.5 was defined as seroconversion.

Human bronchial epithelial cells

Normal human bronchial epithelial cells (NHBE) were obtained from Clonetics (Basel, Switzerland) and used at passage 3–4. Undifferentiated NHBE cells were grown on 30 mg/L type I collagen-coated and 10 mg/L fibronectin-coated 75-cm^2 flasks in serum-free bronchial epithelial cell basal medium supplemented with BEBM SingleQuots (Clonetics). At 60–80% confluency, cells were trypsinized and seeded at a cell density of 1×10^4 viable cells onto type I collagen- and fibronectin-coated 6.5-mm transwell inserts with 0.4-μm pore size (Corning, Amsterdam, The Netherlands) in a 50 : 50 mixture of complete BEBM and Dulbecco's Modified Eagle Medium supplemented with 15 ng/mL retinoic acid. The medium was refreshed every other day until cells reached confluency, then an air-liquid interface (ALI) was created by removing medium from the apical side to promote mucociliary differentiation. The medium was refreshed basolaterally and the apical side was washed with Dulbecco's phosphate buffered saline (DPBS, Lonza, Basel, Switzerland) at 37°C every other day. Well-differentiated (wd-) NHBE cells were used for HMPV infection 21 days after ALI, at which stage beating cilia and mucus production were clearly detectable.

Haemophilus influenzae (ATCC 49247), *M. catarrhalis* (ATCC 25240), *S. aureus* (ATCC 25923) and *S. pneumoniae* (ATCC 49619) were grown twice to log phase on two consecutive days on blood agar plates (*H. influenzae* on chocolate plates). Prior to assays, bacteria were washed two times in antibiotic-free medium. Bacterial numbers were determined by optical density at 600 nm. Recombinant HMPV encoding enhanced green fluorescent protein (EGFP) isolate NL/1/00, further referred to as HMPV-EGFP, was grown as described previously [11] and had a titre of 6.3×10^6 $TCID_{50}$/mL. A recombinant HMPV strain encoding EGFP was used to enable assessment of numbers of infected cells in real-time (i.e. without fixation and immunofluorescent staining).

wd-NHBE cells were washed with DPBS preceding incubation with 50 μL of one of the four different species of bacteria (1.5×10^6 bacteria/mL) for 1 h. After incubation with bacteria, HMPV-EGFP was added (multiplicity of infection

approximately 10, based on an estimated number of 25 000 wd-NHBE cells exposed to the apical surface). Finally, after 1 h incubation with virus, cells were washed twice with DPBS. EGFP-positive cells were counted visually at 24 and 48 h post-infection (h.p.i.).This experiment was performed at least three times using quadruple measurements for each condition.

Statistical analysis

Statistical analyses were performed with SPSS version 15.0. The Fisher's exact test was used for dichotomous outcomes. Mann–Whitney *U*-tests were used to compare differences between mock-treated and bacteria-exposed wd-NHBE cells. $p \leq 0.05$ was considered statistically significant.

Results

Dynamics of IgG antibody levels

Seroconversion to HMPV and MV was measured in healthy children during the first 2 years of life. In cord blood samples, high maternal antibody levels specific for both viruses could be detected, which gradually declined during the first 6 months of life as expected (Fig. 1a, b). All children were vaccinated with a measles-containing vaccine around the age of 14 months, which resulted in seroconversion in all infants at 24 months of age (Fig. 1a). This indicated that all children were immune-competent. At the age of 14 months 18/46 (39%) and at 24 months 29/45 (64%) of the infants had seroconverted to HMPV (Fig. 1b).

Relationship between bacterial colonization and HMPV serology

HMPV seroconversion levels in children frequently exposed to one of the bacterial species (swab culture 2–4 times positive) were compared with those in less frequently exposed children (swab culture 0–1 time positive). Of the total number of children (n = 57), we only had combined seroconversion bacterial exposure data available for 52 children, and thus we limited our statistical analysis to this group. There was no significant association between HMPV seroconversion and exposure to *H. influenzae*, *M. catarrhalis* or *S. aureus* (Table 1, $p > 0.05$). However, 14 out of 15 (93%) children frequently exposed to *S. pneumoniae* had seroconverted to HMPV at 24 months, vs. 22 of 37 (59%) less exposed children (Table 1, $p < 0.05$). In children who remained seronegative for HMPV during the first 2 years of life, only 1 of 16 (7%) was frequently exposed to *S. pneumoniae*. We conclude that children frequently exposed to *S. pneumoniae* mounted a stronger humoral immune response to the virus.

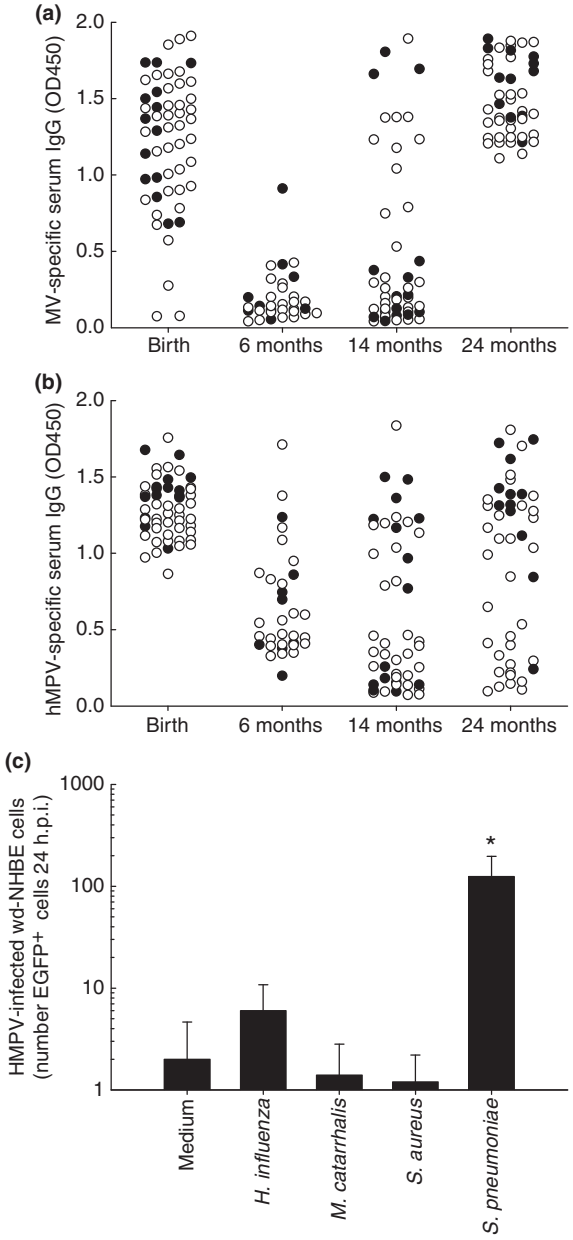

FIG. 1. (a, b) Measles virus (MV)-specific or human metapneumovirus (HMPV)-specific IgG levels in sera of healthy infants collected during the first 2 years of life. Black symbols represent *S. pneumoniae* frequently-exposed children (at least two positive nasal swabs during the 2-year study period), white symbols represent infants less exposed to *S. pneumonia*. (c) Well-differentiated normal human bronchial epithelial (NHBE) cells were incubated with *H. influenzae*, *M. catarrhalis*, *S. aureus* or *S. pneumoniae* (or medium as a control) and subsequently infected with recombinant HMPV encoding enhanced green fluorescent protein (EGFP). Data are plotted as means ± standard deviation of quadruplicate cultures. The experiment was repeated three times. Significant enhancement of HMPV-EGFP infection was seen in wd-NHBE cells pre-incubated with *S. pneumoniae* but not in wd-NHBE cells pre-incubated with any of the other bacterial species. h.p.i. = hours post-infection. $p < 0.05$ (Mann-Whitney U-test)

TABLE 1. Association between frequency of bacterial exposure and human metapneumovirus (HMPV) seroconversion during early life

Bacterial pathogen	Frequent exposure[a]	HMPV seroconversion No: *n* = 16 (31%)	HMPV seroconversion Yes: *n* = 36 (69%)
H. influenzae	No: *n* = 42 (81%)	14 (33%)[b]	28 (67%)
	Yes: *n* = 10 (19%)	2 (20%)	8 (80%)
M. catarrhalis	No: *n* = 41 (79%)	13 (32%)	28 (68%)
	Yes: *n* = 11 (21%)	3 (27%)	8 (73%)
S. aureus	No: *n* = 45 (87%)	14 (31%)	31 (69%)
	Yes: *n* = 7 (13%)	2 (29%)	5 (71%)
S. pneumoniae	No: *n* = 37 (71%)	15 (41%)	22 (59%)
	Yes: *n* = 15 (29%)	1 (7%)	14 (93%)*

[a]Frequent exposure is defined as at least two positive nasal or nasopharyngeal swabs during the 2-year study period.
[b]Data are shown as the number of children, followed by the percentage of those who were or were not frequently exposed between brackets.
*p <0.05 (Fisher's exact test).

S. pneumoniae enhances HMPV infection of normal human bronchial epithelial cells

The observations from the cohort study suggest that exposure to *S. pneumoniae* was associated with seroconversion to HMPV, which provides no evidence for a direct interaction between *S. pneumoniae* and HMPV infections. To test the hypothesis that bacterial exposure could enhance susceptibility to HMPV infection, we used NHBE cells differentiated at the air-liquid interface. Epithelial cells pre-incubated with *S. pneumoniae* were significantly more susceptible to infection with HMPV-EGFP than mock-treated cells, as evidenced by the enumeration of EGFP-positive epithelial cells 24 and 48 h.p.i. (Fig. 1c, $p < 0.05$; data shown for 24 h.p.i.). This was not the case for cells pre-incubated with *H. influenzae*, *M. catarrhalis* or *S. aureus* (Fig. 1c, $p > 0.05$; data shown for 24 h.p.i.).

Discussion

Interactions between viral and bacterial disease are usually interpreted as virus infections predisposing for severe bacterial infections [7,12]. Different mechanisms have been proposed, including virus-induced damage to respiratory cells predisposing to opportunistic bacterial infection or up-regulation of bacterial adhesion molecules by viral infection [13–15]. The aim of the present study was to analyse whether bacterial exposure can influence susceptibility to HMPV infection. First, we demonstrated for 57 healthy children that they were immune-competent; indeed, all children had seroconverted to MV at the age of 24 months, approximately half a year after MMR vaccination. Subsequently, we screened the colonization state of these 57 children for four common respiratory bacterial species (*H. influenzae*, *M. catarrhalis*, *S. aureus* and *S. pneumoniae*) in relation to seroconversion to HMPV during the first 2 years of life. Whereas no relationship was detected between exposure to *H. influenzae*, *M. catarrhalis* or *S. aureus* and HMPV seroconversion, *S. pneumoniae* exposure was significantly associated with increased seroconversion levels to HMPV. These increased HMPV seroconversion levels could be due to increased susceptibility to HMPV infection, increased viral replication or virus spread, or enhanced immune responses to infection.

Differences in bacterial exposure and HMPV seroconversion could also be related to a common external factor (e.g. attendance at a day-care centre). However, in that case we would expect that the carrier state of *H. influenzae*, *M. catarrhalis*, *S. aureus* and *S. pneumoniae* would also be affected. On the basis of the serology data, we concluded that either HMPV infection leads to more frequent *S. pneumoniae* carriage or exposure to *S. pneumoniae* increases the susceptibility to HMPV infections.

To test our hypothesis that bacterial exposure could increase the susceptibility to HMPV infection, we used wd-NHBE cells cultured on the air-liquid interface. The cells clearly showed beating cilia and mucus production, and may therefore be considered the best possible *in vitro* mimic of the target cells of HMPV infection *in vivo*. Surprisingly, apical infection of wd-NHBE cells with HMPV-EGFP at an estimated MOI of 10 resulted in a low frequency (<1%) of EGFP-expressing cells 1–2 days after infection. Apparently, apical HMPV infection of wd-NHBE cells is a relatively inefficient process. However, pre-incubation of the wd-NHBE cells with *S. pneumoniae* resulted in increased susceptibility to HMPV infection as compared with mock controls, as evidenced by more than ten times higher numbers of infected cells (Fig. 1c).

Several mechanisms could contribute to the observed increased seroconversion levels in children, or the increased numbers of *in vitro* infected wd-NHBE cells, after exposure to *S. pneumoniae*. Pneumococcal virulence and immune evasive factors, including capsule or pneumolysin, may facilitate HMPV infection in multiple ways. First, these factors may penetrate the mucus layer and inhibit ciliary beating of respiratory epithelial cells [16]. This could expose susceptible human epithelial cells, resulting in enhancement of HMPV infection or spread. In addition, HMPV infection may be facilitated by influx or activation of immune cells (neutrophils, lymphocytes or dendritic cells) residing in or associated with the respiratory epithelium [16]. Moreover, bacterial factors stimulating TLR2 and TLR4 responses may provoke an enhanced immune response following HMPV infection. Furthermore, lipopeptides in the bacterial cell wall may lead to enhanced viral binding to target cells [17], facilitating HMPV infection and spread. In addition, pneumococcal immune

evasive factors counteract host innate immune responses, which may also facilitate HMPV infection.

In conclusion, our combined *in vivo* and *in vitro* data suggest a specific interaction between *S. pneumoniae* and HMPV infection.

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Transparency Declaration

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The authors declare that they have no conflicts of interest.

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